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Derivation of androgenetic embryonic stem cells from *m*-carboxycinnamic acid bishydroxamide (CBHA) treated androgenetic embryos

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The androgenetic embyronic stem (aES) cells are useful models in studying the effects of imprinted genes on pluripotency maintaining and embryo development. The expression patterns of imprinted genes are significantly different between uniparental derived aES cells and zygote-derived embryonic stem (ES) cells, therefore, the imprinting related cell pluripotency needs further exploitation. Several approaches have been applied in generation of androgenetic embryos and derivation of aES cell lines. Here, we describe a method to generate androgenetic embryos by injecting two mature sperms into one enucleated oocyte. Then these androgenetic embryos were treated with a histone deacetylase inhibitor: *m*-carboxycinnamic acid bishydroxamide (CBHA). Further, aES cell lines were successfully derived from these treated androgenetic embryos at blastocyst stage. The CBHA could improve not only the quality of androgenetic embryos, but also the efficiencies of aES (CaES) cells derivation and chimeric mice generation. The imprinted gene expression pattern in the CBHA treated embryo-derived aES (CaES) cells was also highly similar to that of zygote-derived ES cells.

CBHA, androgenetic, aES, pluripotency

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Normal sexually reproductive embryos obtain paternal and maternal genomes equally. Diploid androgenetic and parthenogenetic embryos have two sets of uniparental genomes and they can only develop to somite or limb buds stage [1–3] due to lacking of the parental genome and having different expression pattern of imprinted genes compared with fertilized zygotes [4]. About 30 years ago, pronuclear exchange between two zygotes was mainly used for producing androgenetic embryos [5,6], but the reconstruction process was complicated. Recently, somatic cell nuclear transfer (SCNT) method has been applied to reconstruct androgenetic embryos by injecting two sperms or two round sperms into enucleated oocytes, which improved the reconstruction efficiency apparently [7,8]. Although androgenetic embryos can not develop to term, aES cells can be derived from the inner cell mass (ICM) of androgenetic blastocysts generated by these two methods. The aES cells had typical mouse ES domed colonies [9] and expressed pluripotent markers (such as *Oct4*, *Nanog* and *Sox2*), however, the expression pattern of imprinted genes in them was quite different from that in zygote-derived ES cells, which resulted in hardly generation of healthy chimeric mice [10,11]. Even one lab reported aES cells got germline competency, nobody in other labs could repeat their results so far [12]. Several groups have reported androgenetic embryos could be generated from

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different spermatogenic stages' donor cells (primary spermatocyte, round spermatids or mature sperms). These androgenetic embryos had different developmental potentials and their corresponding aES cells showed great different pluripotency [8,11]. Up to date, there have no healthy chimeric mice been generated from two mature sperms-derived aES cells assisted with SCNT method, which might be due to the incomplete reprogramming of the mature sperms in enucleated oocytes. When two sperms were injected into one enucleated oocyte, fast activation of demethylation occurred in the paternal genomes [13] and epigenetic modification could be introduced by multiple environmental factors [14,15]. CBHA could change global epigenetic status of SCNT embryos and improve their developmental abilities [16], therefore, we examined the effect of CBHA treatment on the mature sperm's reprograming efficiency.

In this study, we reconstructed androgenetic embryos from two mature sperms by using classic nuclear transfer technology-"one step micromanipulation" (OSM) [17] efficiently and treated them with CBHA during activation process. Thereafter, we derived aES cell lines from these androgenetic embryos. These aES cell lines could generate healthy chimeric mice by 4- or 8-cell embryos microinjection. This process could not only supply a source of histocompatible stem cells for cell transplanted-based therapies [18] but also develop a unique model to study imprinted gene expression pattern.

1 Materials and methods

1.1 Mice

Specific pathogen-free (SPF) grade mice were purchased from Beijing Vital River Laboratory Animal Center and housed in the animal facility of the Institute of Zoology. All studies were performed in accordance with the Guidelines of Institute of Zoology, Chinese Academy of Sciences for the Use of Animals in Research.

Male 129S2/SvPasCrl (129Sv as the short name) Female B6D2F1 (C57BL/6J×DBA/2) mice and CD-1 mice.

1.2 Reconstruction of androgenetic embryos and CBHA treatment

Androgenetic embryos were reconstructed with "OSM" method [17]. MII oocytes were collected from the super ovulated 8-week-old B6D2F1 female mice. With piezo's assistance, two sperm heads were microinjected into one enucleated oocyte under M2 medium (Sigma) which contained 5 μg mL^{-1} cytochalasin B (CB). The reconstructed embryos were cultured in KSOM medium supplemented with 5 μg mL^{-1} of CB and CBHA (Calbiochem, working solution 20 μmol L^{-1}) for 6 h and then transferred to KSOM medium at 37°C, 5% CO_2.

1.3 Derivation of androgenetic embryonic stem cell lines

Androgenetic embryonic stem cell lines were derived as previously described [19]. Blastocysts were transferred to gelatinized 4 multi-well plate coated with mitomycin C-treated murine embryonic fibroblast feeder (MEF) cell layers in ES medium. Inner cell mass derived outgrowths were mechanically dissociated into clumps gently and replaced on fresh feeder layers in ES culture medium, designated as P1. When typical ES cell colonies appeared on the feeder layer, the culture medium is replaced by the "2i" medium [20]. All cell lines were passaged every two days.

1.4 Alkaline phosphatase staining, immunocytochemistry and Western blot

Alkaline phosphatase staining was performed using the BeyoAP Alkaline phosphatase kit (Beyotime) according to the manufacturer's instructions. For immunocytochemistry assay, aES cells were fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized in 0.5% Triton X-100. Samples were incubated with mouse monoclonal primary antibody Oct3/4, Nanog, Sox2 and SSEA-1 (Chemicon) at 4°C overnight. After washing in PBS, samples were incubated with Alexa Fluor488-conjugated anti-mouse secondary antibodies at room temperature for 1 h and 50 μg mL^{-1} of PI was used for staining the nuclei. Images were captured on a confocal microscope (ZEISS). For Western blotting, protein samples were extracted from CaES cells, MEF cells and R1 ES cells. Electrophoresed samples were transferred onto a polyvinylidene fluoride (PVDF) membrane. Blocked membranes were incubated with primary mouse monoclonal anti-Oct3/4 (Santa cruz) antibody, rabbit polyclonal anti-Nanog (Abnova) at 4°C overnight. HRP conjugated anti-mouseantibody, anti-rabbit antibody (1∶1000 dilution; WAKO) was added to the washed membranes. Immunoreactivity was visualized by an ECL detection kit (Millipore) and imaged by program Quantity One (Bio-Rad).

1.5 Karyotype analysis

Standard G-banding karyotype analysis of aES cells were performed by Peking Union Medical College, Beijing.

1.6 Teratoma formation assay

For each cell line, about 2×10^6 aES cells were subcutaneously injected into the hind limb of a 6-week-old male SCID Beige mouse. Fully formed teratomas were dissected and fixed with 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin-eosin for histological analysis.

1.7 Chimera production and contribution analysis

Two-cell stage embryos were collected from superovulated female CD-1 mice (1.5 days post coitum [d.p.c]) and cultured to 4- or 8-cell stage in KSOM medium. Around 12–15 aES cells were microinjected into each embryo [21]. Manipulated embryos were transferred into the oviduct of 0.5 d.p.c pseudo-pregnant CD-1 mice. Coat color and SSLP analysis [11] were applied to evaluate the contribution of aES cells in chimeras. Primers used for SSLP were listed in Table S1.

1.8 Real-time PCR

Trizol (Invitrogen) with DNase I was used to extract total RNA from CaES, aES, R1 and pES cells with about 1×10^5 cells for each sample. All the samples were reverse transcribed to cDNA with the MLV system (Invitrogen). Real-time PCR was performed to analyze relative quantitation of imprinted genes of these cell lines with SYBR Green PCR Master Mix (Toyobo) according to the manufacturers' instructions. Amplification data were collected by the Rotor-GeneQ (QIAGEN) and analyzed by the Rotor-Gene Q software (QIAGEN). The *Gapdh* gene was used as the endogenous control and each assay was carried out in triplicate. All the primers were listed in Table S1.

1.9 DNA methylation analysis of DMRs in imprinted genes

Genomic DNA was extracted from each set of cultured ES cells using DNA extraction buffer. The DNA was subjected to bisulfate modification with the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. The bisulfite converted DNA was amplified by nested (semi-nested) PCR as previously described [22]. Detailed primer sequences were listed in Table S1. PCR products were subcloned into a pGEM-T Easy vector (Transgene), and then sequenced by M13 reverse primers for each DMR.

1.10 Statistical analysis

The Analysis of variance (ANOVA) was used for statistical analysis. For all statistical analyses, a value of $P<0.05$ was considered to be statistically significant.

2 Results

2.1 Derivation of aES cell lines from CBHA treated androgenetic embryos

Androgenetic embryos were successfully generated from two matured sperms by "OSM" method (Figure 1(a)). These

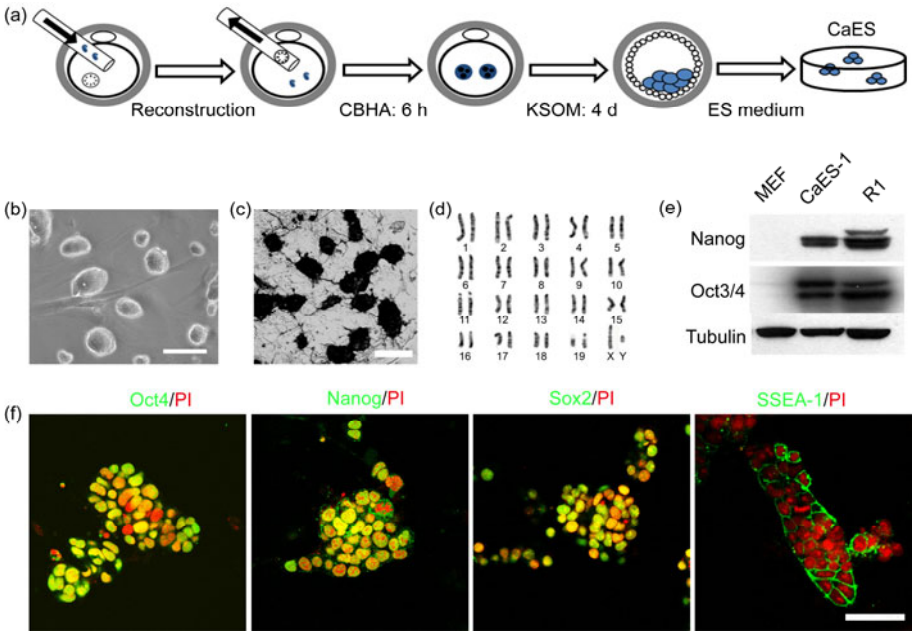

Figure 1 Derivation of aES cell lines from CBHA treated androgenetic embryos. (a) The schematic representation of deriving CaES cell lines. Two sperm heads were microinjected (129Sv genetic background) into one enucleated MII oocyte (B6D2F1 genetic background) to reconstruct an androgenetic embryo. These embryos were cultured in KSOM medium supplemented with Cytochalasin B (CB) for avoiding polar body extrusion and with CBHA for modifying the genomic epigenetic state. Six hours later, activated embryos were transfer to KSOM medium for cleavage until blastocyst stage. All these treated androgenetic blastocysts were plated on feeder layers in ES medium to derive aES cell lines. (b) Morphological colonies of CaES cells (cell line-CaES-1 passage 10), bar=100 μm. (c) Alkaline phosphatase (AP) staining. All colonies of CaES-1 (passage 10) were strongly positive, bar=100 μm. (d) Standard G-banding karyotype analysis of CaES cells. CaES cells (CaES-1) present a normal 40, XY karyotype. (e) Western blotting analysis of pluripotent markers in a CaES cell line (CaES-1 passage 12). Mouse ES cell line R1 as positive control, murine embryonic fibroblast (MEF) cells as negative control and tubulin as loading control. (f) Immunostaining of pluripotent markers in CaES cells (CaES-1 P15): Oct4, Nanog, Sox2 and SSEA-1 were on FITC channel (green), DNA was marked with propidium iodide (PI, red), bar=50 μm.

embryos were activated by the sperm heads without chemical compound's ($SrCl_2$) stimulus. Meanwhile, CB was used for keeping the embryos diploidy and CBHA was used for promoting reprograming. The CBHA treated androgenetic embryos showed a higher blastocyst rate (18.1%) than non-treated ones (9.9%) (Table S2). Subsequently, two CBHA treated embryo-derived aES cell lines (CaES-1 and CaES-2) and two non-CBHA treated embryo-derived aES cell lines (aES-1 and aES-2) were established (Table S2).

2.2 Characterization of aES cells

CaES cells were morphologically similar to normal ES colonies (Figures 1(b) and S1) and had strongly positive activity of alkaline phosphatase (AP) (Figures 1(c) and S1). G-banding karyotype analysis showed all CaES cell lines presented normal karyotypes of 40, XY (Figure 1(d)). Validated XX or YY aES cell lines could not be obtained. The expression pattern of pluripotent markers, such as *Oct4*, *Sox2*, *Nanog* and *SSEA1* in CaES cells was similar to that of normal ES cells (Figure 1(e) and (f)). Due to having two sets of paternal genomes in aES cells, we further detected the expression pattern of imprinted genes and DNA methylation state in differentially methylated region (DMR) of CaES cells. Two paternal genes (*Snrpn* and *Igf2*) showed a higher expression level in CaES cells than that in R1 ES cells (zygote-derived ES), whereas, two other paternal genes (*Dio3* and *U2af1-rs1)* had similar expression level with R1 ES cells'(Figure 2(a)). The expression level of maternal genes *Asb4*, *Gtl2* and *Meg3* except for *H19* in CaES cells was lower

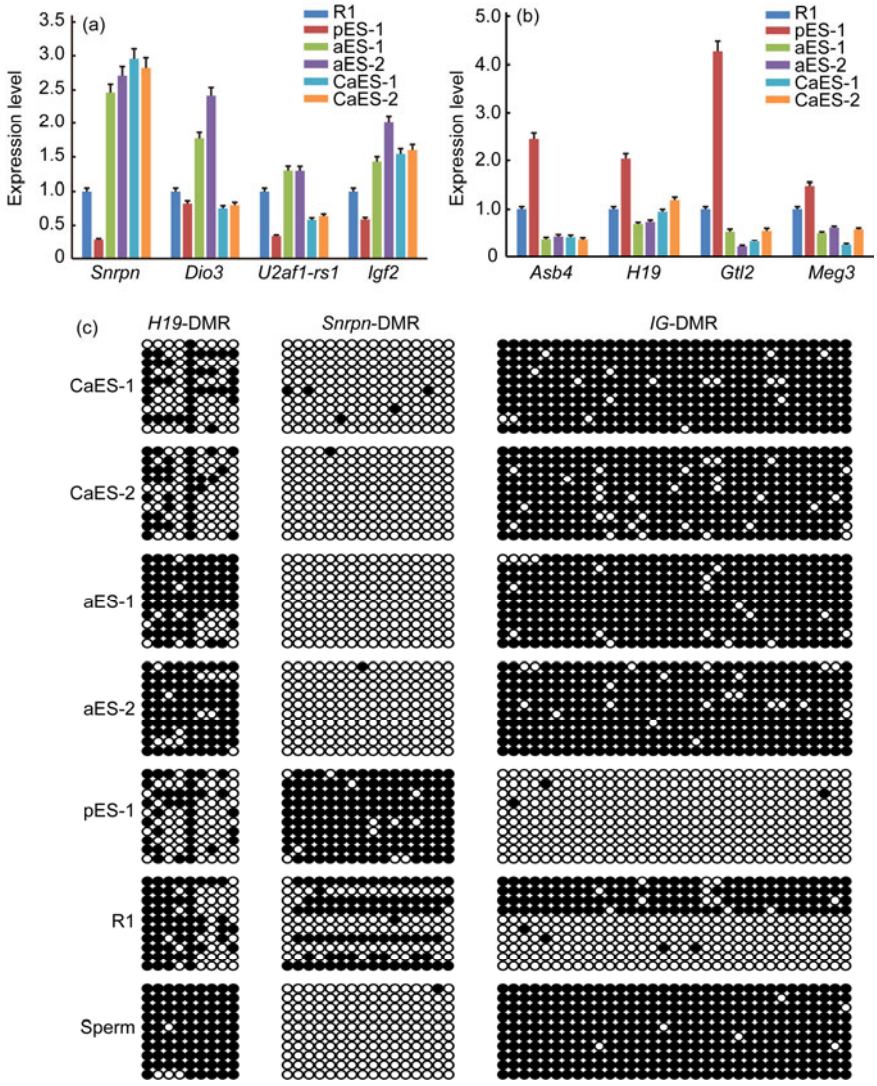

Figure 2 Expression levels of imprinted genes and the methylation pattern of DMRs in CaES. (a, b) Expression level of imprinted genes in CaES cell lines. Conventional ES cell line R1 was used as control. Endogenous control Gapdh was applied to normolize the loading mRNA. Error bar indicated standard deviation (n=3). All the ES cells were controlled at passage 15. (a) Expression levels of paternal genes in zygote-derived ES (R1), parthenogenetic ES (pES-1), non-treated aES (aES-1 and aES-2) and CaES (CaES-1 and CaES-2). (b) Expression levels of maternal genes. (c) Methylation paterns of CaES cell lines. Bisulfite genomic sequencing analysis of DMR of imprinted genes *Snrpn*, *H19* and *IG*. Filled circles, methylated CpG sites. Empty circles, unmethylatedCpG sites. All the ES cells were controlled at passage 15, sperms were collected from adult 129Sv strain male mice.

than in R1 ES cells (Figure 2(b)). According to the bisulfite sequencing results, CaES maintained the uniparental feature in *Snrpn*-DMR, *IG*-DMR, but lost imprinting in *H19*-DMR (Figure 2(c)), which might account for their improved chimeric competency.

2.3 Pluripotency of CaES cells *in vivo*

CaES cells could form typical teratoma 3 weeks after injection into immune deficient mice. Histological analysis confirmed that CaES cells could differentiate to gland (endoderm), muscle (mesoderm) and epidermis (ectoderm), which indicated CaES cells had differentiation potentials to form three germ layers *in vivo* (Figure 3(a)).

We reconstructed 866 chimeric embryos from two CaES cell lines and gained 19 chimeras. Compared to non-treated aES, CaES had higher chimeric competency as described in Table 1. CaES cells contributed to majority tissues (CaES-1, 9 tissues) of the chimeras, identified by coat color and SSLP analysis (Figure 3(d) and (e)). Whereas, the aES cells only contributed to minority (aES-2, 4 tissues) tissues (Figure 3(b) and (c)).

3 Discussion

Similar to normal ES cells, the aES cells will become an alternative resource for clinical histocompatible cell therapies

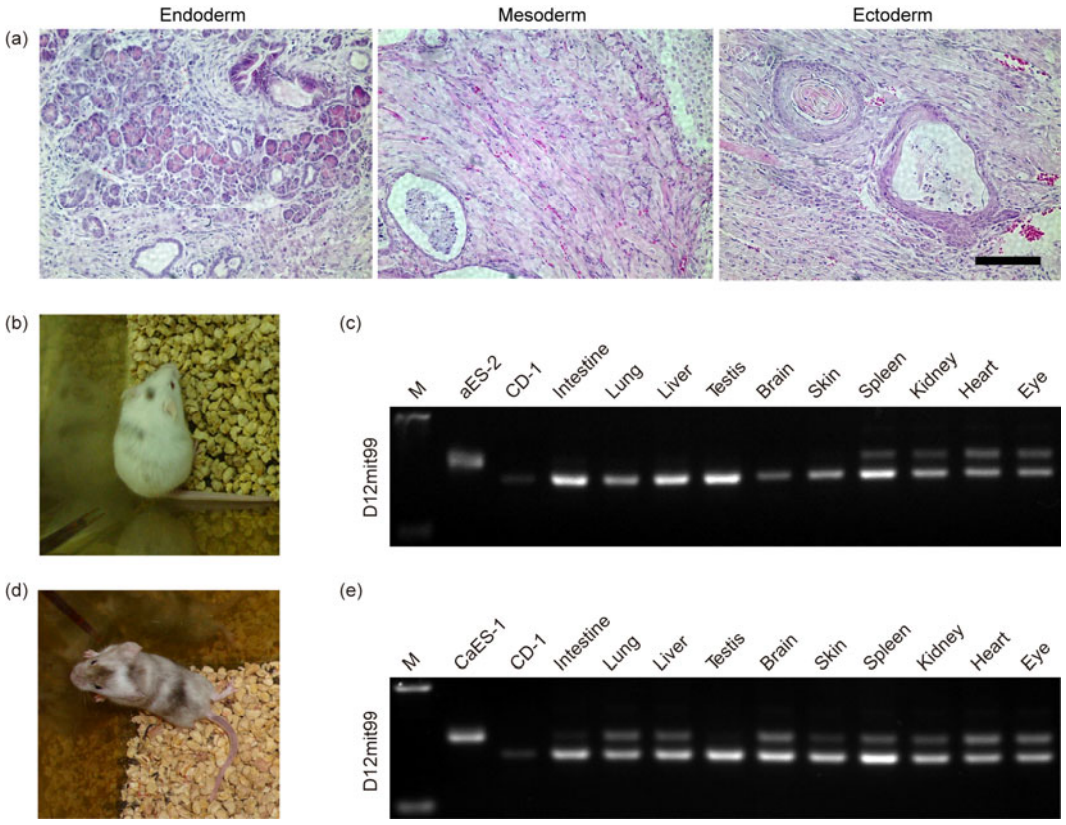

Figure 3 Pluripotency of CaES cells *in vivo*. (a) Histological analysis. CaES-1 cells could differentiate into three germ layers by teratoma formation assay. Left was gland (endoderm), middle was muscle (mesoderm), right was skin (ectoderm). (b) Chimera generated from CBHA non-treated aES cells (cell line: aES-2 passage 12). (c) SSLP analysis of contribution of aES cells in chimera showed in panel B. (d) Chimera generated from CBHA treated aES(CaES) cells (cell line: CaES-1 passage 10). (e) SSLP analysis of contribution of CaES cells in chimera showed in panel (d).

Table 1 Summary of chimeras generated from CaES cell lines

Cell line[a)]	No. of embryos manipulated	Pups		Chimera (%)[b)]
		No. of abnormal	No. of normal	
CaES-1	421	4	77	11(2.6±0.9)[a]
CaES-2	445	2	63	8(1.8±0.3)[a,b]
aES-1	414	4	36	2(0.5±0.8)[c]
aES-2	340	6	41	4(1.2±0.5)[a,b]

a) All the cell lines used for chimeric assays were lower than passage 20. b) All the efficiencies (shown as %) were calculated based on the total manipulated embryos. Values with different superscripts are significantly different in one column by one-way ANOVA, $P<0.05$.

and this type of ES cells will be a valuable tool for epigenetic regulation. The difficulty of generation healthy chimeras from uniparental aES cells by SCNT was might lie in lacking of maternal genome and strict paternal imprinting. Although SCNT method could simplify the reconstructing process, it could not erase the strict paternal imprinting of aES cells. Most of the chimeras from aES cells had skeletal abnormality and other defects [10,23], and no chimeras were generated from two mature sperms derived aES cells assisted with SCNT previously. Some labs tried to erase the strict imprinting of uniparental ES cells by serial nuclear transfer method [24] or long-term cell culture [25]. But these methods were either complicated or time-consuming. Here, we found CBHA, a small compound, could make aES cells more similar to normal ES than non-teated aES cells in imprinted gene expression. Two paternal and one maternal genes in CaES cells have been inversed to the normal level (Figure 2(a) and (b)), which could be a good explanation that CaES cells have competency to generate healthy chimeras. Three imprinted genes *H19*, *Dio3* and *U2af1-rs1* which play important roles in neural system, immunity and organ development [26] could also be regulated by histone modification [27,28]. CBHA might change imprinted genes' DMR methylation status by global histone modification in the process of androgenetic embryos treatment before pronuclear stage. Naturally, it was abnormal that two or more sperms existed in one oocyte, especially when artificial assisting the process, these embryos as SCNT embryos had poor developmental ability [23,29]. Epigenetic modifications in imprinted genes play an important role in development potential of uniparental embryos and pluripotency of their stem cells [30,31]. As a histone deacetylase inhibitor, CBHA could adjust acetylation sites and DNA methylation in the process of SCNT embryos' development, which was similar to that happened in zygotes. CBHA could not only increase fetus rate of cloned embryos, but also improve the derivation efficiency of NT-ES cell lines [16,32]. In this study, we found that CBHA could improve pluripotency of androgenetic embryo-derived aES cells. Recently, the androgenetic and parthenogenetic technologies have been used in producing haploid stem cells which is a brand-new research spot and have great application potentials in recessive traits and assisted reproductive medicine [33–36]. However, the efficiency of cell line derivation and stability of haploid stem cells also need to be tackled [36,37]. Epigenetic abnormalities were also detected in retarded pups generated from intracytoplasmic androgenetic haploid stem cell injection (ICAI) [36,37]. Through understanding of aES cells may help solving some issues puzzling haploid embryonic stem cells researchers.

4 Conclusions

In summary, CBHA treated aES (CaES) cell lines can be derived from androgenetic embryos treated with CBHA at early stage and they can generate healthy chimeras. Although CaES cells need further optimization to gain pluripotency like zygote-derived ES, the distinction of imprinted gene expression pattern in CaES cells from previous aES cells open a new dimension for epigenetic regulation research.

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Supporting Information

Figure S1 Immuno- and AP- staining of normal ES cells (R1) and non-treated aES cells (aES-2).
Table S1 Primers for PCR
Table S2 *In vitro* development of reconstructed androgenetic embryos with or without CBHA treatment

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